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TITLE: Breast Tumor Kinetics in Mice Overexpressing Cyclin E
and Heterozygous for Tumor Suppressor p53 or Rb

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Table of Contents

| | |
|-----------------------------------|----|
| Cover..... | 1 |
| SF 298..... | 2 |
| Table of Contents..... | 3 |
| Introduction..... | 4 |
| Body..... | 4 |
| Key Research Accomplishments..... | 8 |
| Reportable Outcomes..... | 8 |
| Conclusions..... | 9 |
| References..... | 9 |
| Appendices..... | 13 |

Introduction

The purpose of this project was to test whether deregulated cyclin E in the mammary epithelia predisposes to chromosomal instability and hence tumorigenesis. To test this hypothesis, based on in vitro work (Spruck et al. 1999), we generated transgenic mice expressing either wild-type human cyclin E or a hyperstable mutant (T380A) with mice heterozygous at either the p53 or Rb loci. If genomic instability were induced by deregulated expression in the mammary epithelia, we anticipated an increased penetrance and decreased latency of tumorigenesis. Neither heterozygosity of p53 or Rb predisposes to mammary tumorigenesis in mice (Jacks et al. 1992; Jacks et al. 1994; Harvey et al. 1995) except in the BALB/c background (Kuperwasser et al. 2000). The evaluation of this model has involved the integration of molecular and pathological analysis.

Body

All technical objectives outlined for this project have been met.

Technical objective 1.

Task 1. Months 1-2.

Task 2. Months 2-6.

Technical objective 2.

Task 3. Months 5-36.

Task 4. Months 8-36.

Technical objective 3.

Task 5. Months 1-6.

Task 6. Months 12-36

Generation of founder mice

Cross breed transgenic and heterozygous strains.

Genotype offspring and identify mice appropriate as founders.

Characterization of tumor kinetics and types

Weekly examination by palpitation and recording approximate tumor size.

Terminal necropsy, tumor collection and histopathology.

Genomic instability

Establishment of primary cell culture and chromosome painting techniques.

Karyotype analysis of cells derived from 'normal' and tumor mammary specimens from all genotypes.

Overview

At the conclusion of the study it is clear that there is a synergy between p53 heterozygosity and deregulated expression of a hyperstable allele of cyclin E in mammary tumorigenesis in the mouse model developed. Furthermore, while the tumors that developed were clearly aneuploid, genetically unstable and showed loss of p53 heterozygosity, the vast majority retained transgene expression as determined by immunohistochemistry and by western blot analysis of serially cultured cells. This implies that the hyperstable cyclin E transgene may confer a growth advantage both in vitro and in vivo. However, while the anticipated increase in mammary tumor penetrance has been observed, a concomitant decrease in latency was not. This is thought to be due to the unique feature of the mammary gland, i.e. the mammary epithelia involute post lactation and genetic lesions induced may be largely lost. While all analysis as described in the original proposal has been completed our current efforts are to demonstrate this early loss of heterozygosity utilizing interphase cytogenetics, a technique not originally anticipated. Completion of this undertaking and submission of the manuscript for publication (currently being prepared for *Cancer Cell*) is realistically anticipated within 2 months of this report. A study of this size has generated a large amount of data and a comprehensive account is prohibitory for this report. The main manuscript is in preparation and several shorter supplementary manuscripts are anticipated on incidental findings as case reports. A possible correlation between mammary tumorigenesis and parity in these mice which is currently being statistically evaluated may also lead to a further manuscript. We include here a synopsis of the major findings pertinent to the original project outline and the main manuscript, excluding incidental data which has already lead to one publication (see report 2002) and findings related in previous reports .

Mammary tumor incidence and latency.

Although some mice failed to meet the strict inclusion criteria or met exclusion criteria set at the beginning of the study, sufficient mice for statistical analysis were obtained in all genotypes. Final confirmation of diagnoses are underway as Dr John Lee is currently in San Diego (May27-June1,03). A summary of the tumor incidence is presented in Table 1. and tumour latency in Fig 2. *Inclusion and exclusion criteria.* All mice evaluated in the study were set up in breeder cages for two pregnancies to induce the transgene (or comparable parity in non-transgenic mice). Transgenic mice were only included only if homozygous at the transgene locus. Mice were excluded from further analysis if they met one of the following criteria; i) <2 pregnancies, ii) premature euthanasia or death from non-neoplastic lesions, iii) premature death or euthanasia prior to the appearance of the first mammary lesion observed in any of the genotypes (see appendix).

Cyclin E transgenic mice, transgene zygosity and expression.

Both the cyclin E and hyperstable T380A transgenic alleles were inherited independently of both the p53 and Rb loci and as a single locus. Furthermore, each transgene segregated equally with gender and was therefore not located on either X or Y chromosomes. Genotype did not alter expected heredity patterns, the age of sexual maturity or litter size. The strains generated were homozygous or nullizygous at the transgene locus and wild-type or heterozygous at the tumor suppressor loci. The nine strains generated were designated as wild-type, CycE, T380A, Rb^{+/-}, Rb^{+/-}CycE, Rb^{+/-}T380A, p53^{+/-}, p53^{+/-}CycE and p53^{+/-}T380A (see previous reports). Immunohistochemical and western analyses were performed on mammary tissue from midlactation (2 week post-partum) and post-lactation (6 and 8 weeks post-partum) mice. HE12 immunoreactivity (human cyclin E only) was detected in the nuclei of the mammary epithelia in the 2 week post-partum females but not in the 6 week post-partum females. Immunoreactivity was negative in all other tissues examined in the midlactation females which included all thoracic and abdominal viscera, central and peripheral nervous tissue, haematopoietic tissue, skeletomusculature and integumentary.

When examined by HE12 immunohistochemistry, virtually all the mammary epithelia showed transgene expression but the more intense staining cells also appeared to be those either in hyperplastic projections and/or with enlarged nuclei. Measurement of nuclei size however showed a significant decrease of approximately 30% in the average size of nuclei in midlactation mammary glands of mice bearing the T380A allele (table 3). Quantitation of protein levels in tissue by immunohistochemistry is problematic, so comparisons between lines were performed by immunoblot analysis showing higher levels of transgene expression in the mammary tissue of T380A lines with slightly lower but still appreciable expression in the strains transgenic for the wild-type allele (Fig 1) expressed as two forms at 47 and 35 KDa. The two forms represent alternative translational start sites rather than proteolytic products. We noticed however that the ratio of the 47Kda product to the 35kDa band is roughly equivalent in the mice transgenic for the wildtype cyclin E allele while the faster migrating band is more prevalent in the T380A transgenic mice. Titration of the T380A signal against that from cyclin E mice showed a 5-20 fold higher expression dependent upon comparison of the 47 or 35KDa products. Analysis of transgene dependent H1-kinase activity demonstrated that while there was no transgene dependent kinase activity in the cycE mice, those in the midlactation mammary glands of T380A mice were elevated.

The non-mammary tumor spectrum in Rb^{+/-} or p53^{+/-} mice is unaffected by cyclin E transgenes

The tumor spectrum anticipated in our mice broadly falls into three categories; 1) those induced by the cyclin E transgene, 2) those induced by tumor suppressor heterozygosity and 3) background lesions expected in all aged mice. The last category is largely obviated in this study

as a final time point of 18 months precludes the majority of these lesions. Nevertheless, some background lesions were observed none of which achieved a statistical significance in this study (Table 1). The spectrum of tumor susceptibility in $Rb^{+/-}$ and $p53^{+/-}$ mice is well established although we are not aware of a systematic study of female mice for either strain. In both strains, we observed a tumor spectrum similar to those of published reports (Lee et al. 1992; Jacks et al. 1994; Harrison et al. 1995; Harvey et al. 1995; Park et al. 1999). The predominant lesions in $Rb^{+/-}$ mice were pituitary tumors and medullary thyroid carcinomas with frequencies comparable to those in the literature. Likewise, $p53^{+/-}$ mice exhibited a similar sarcoma tumor spectrum to that published with osteosarcomas, lymphomas and poorly differentiated sarcomas frequently represented. In addition we observed a moderate rate of mammary carcinoma and pituitary pars distilis carcinoma, the latter of which were always accompanied by galactorrhea. Leiomyosarcomas were more frequent in $p53^{+/-}$ mice than either $Rb^{+/-}$ mice or mice without mutations at either locus but were observed as infrequent uterine lesions in all the strains studied, including wild-type control and transgenic strains and those meeting exclusion criteria. When either the hyperstable or wild type allele of cyclin E was also present the tumor suppressor spectrum of tumors was similar to those without the transgene. This is indicative that there was no significant alteration of tumor spectrum other than a decrease in osteosarcomas (category 2 above) in the $p53^{+/-}$ T380A mice which was presumably due to the increased incidence of mammary tumors (category 1) in these mice.

Mammary tumors were not observed in any strain of mice bearing an Rb mutation. Pituitary adenomas were a frequent lesion encountered in all three $Rb^{+/-}$ strains and were frequently the cause for premature euthanasia. Although the lesions were always histologically consistent with cells of a pars intermedia origin, several mice exhibited excessive mammary development at necropsy, even to the point of overt galactorrhea in some cases, suggestive of prolactinaemia. Prolactin immunohistochemistry proved largely negative in these lesions although a few lesions demonstrated a weak diffuse positive staining in some tumor cells. A common theme to the cases of galactorrhea were a seemingly functional pars distilis, often completely ablated in the larger tumors, with an intermediate sized pars intermedia lesion (3-4mm in the longest dimension). We interpret these cases as a destruction of the hypophyseal stalk and consequently the normal regulatory suppression of prolactin secretion by prolactin inhibitory factor from the hypothalamus is lifted. Nevertheless, even in these cases of overt prolactinaemia, mammary tumors were not observed with or without the presence of a prolactin sensitive cyclin E transgene. The age of onset, frequency, histological grade and morphology were comparable between each of the three $Rb^{+/-}$ bearing mouse strains. Similarly, medullary thyroid carcinomas were observed in approximately 75% of mice with Rb mutations. Clinical morbidity due to thyroid tumors was an infrequent complication. However in some cases mice exhibited involuntary vocalization due to compression of the trachea. Immunohistochemistry demonstrated consistently positive calcitonin immunoreactivity and negative transgene immunoreactivity. A manuscript for an interesting incidental finding of hyperpigmentation in some of these mice is attached.

p53 heterozygosity acts synergistically with T380A to promote mammary tumorigenesis and decreases life expectancy

Surprisingly, none of the CycE mice in the present study developed neoplastic lesions which is most likely due to the greater representation of the C57BL/6J genetic background in the mice of the present study, which are generally less susceptible to mammary tumors than C3H mice. Even though the tumor spectrum was unaltered in mice bearing the $p53^{+/-}$ mutation with or without cyclin E transgenes, the frequency with which they presented was markedly changed in the presence of the hyperstable cyclin E transgene. The frequency of mammary tumorigenesis in $p53^{+/-}$ mice without a transgene was 7% (3/45). In mice also with the wildtype cyclin E transgene this was unaltered at 6% (2/32). However, in mice bearing the hyperstable allele of cyclin E the

penetrence was increased to >50%. This effect was significantly greater than any additive effect as the mammary tumor frequency in the transgenic mice for the hyperstable allele in the absence of a p53 mutation was merely 12% ($p=0.0008$; Fisher's exact test). However, the age of onset was not significantly changed (Fig 2). Three p53^{+/-} mice (8%) developed mammary adenocarcinoma before 18 months with the earliest manifestation observed at 13 months. The earliest age at which any mouse presented with a mammary carcinoma was 10.8 months months.

The mammary tumours of this study did not show a single distinctive growth pattern. Indeed numerous well identified growth patterns were usually observed within each mammary tumor consitent with those seen in spontaneous lesions and the spectrum of growth patterns reported in p53^{+/-} mice. Histologically, most of the mammary tumors were composed of dense sheets or nests of neoplastic cells usually interspersed with fine trabeculae and foci of squamous differentiation. Nuclear/cytoplasmic ratios, nuclear atypia, necrosis and mitotic rates were high. Local invasion of the overlying integument, underlying musculature or surrounding fat pad were a frequent observation. However, despite the poor differentiation and obvious malignant biological behavior, hematogenous metastases were infrequently observed, despite routine histological examination of lung, liver and marrow. Lymphatic dissemination was observed in several cases with large lesions but was a generally infrequent observation. Carcinoma in situ were observed in three p53^{+/-}T380A mice. All tumours were diagnosed as high grade on the basis of mitotic index and pleomorphism and intermediate to high grade on cellular differentiation. However, the lesions considered intermediate in terms of acinar growth patterns or intermediate squamous differentiation demonstrated malignant biology with local invasion.

That the incidence of mammary tumourigenesis overlays the normal tumor spectrum in p53^{+/-}T380A mice is evident in the paucity of tumor free mice at 18 months. Three of these mice developed two mammary lesions, which were presumed to be independent of each other as in each case the lesions were comparable in size with differing growth patterns and in contralateral glands. Furthermore, most mice baring mammary lesions also bore neoplastic lesions in other tissues. Indeed, one p53^{+/-}T380A mouse presented with 4 independent neoplastic lesions; 2 mammary carcinomas in an inguinal and contralateral abdominal gland, splenic lymphoma and a large poorly differentiated sarcoma of a mediastinal lymph node. Multiple lesions were observed in p53^{+/-} mice but at a lower frequency.

Mammary tumors in p53^{+/-}T380A mice tended to present as aggressive fast growing lesions. Typically a size of 1cm was reached within 2-5 days of the lesion being first detected whereas lesions in T380A or p53^{+/-} mice took 3-8 days. Furthermore, several of p53^{+/-}T380A lesions exhibited extraordinarily high mitotic indices, >30 mitotic figures per 40x objective field, and large areas of necrosis. By contrast lesions of T380A or p53^{+/-} mice were typified by more moderate mitotic rates of 4-15 figures per 40x objective field. In F1 generation p53^{+/-}T380A mice, where each mouse is heterozygous at the locus of transgene insertion, mammary tumourigenesis was observed at an equivalent rate; 60% (3/5) indicating that the level of expression may not be the decisive factor, rather the period of expression.

Survival curves of p53^{+/-}T380A mice showed a mildly reduced life expectancy over both p53^{+/-} and T380A mice. Whereas approximately 20% of p53^{+/-} and 75% of T380A mice were tumor free at 1.5 years, no p53^{+/-}T380A animal reached this age tumor free.

Mammary tumors show loss of p53, aneuploidy and constitutive expression of the cyclin E transgene

Analysis for loss of p53 heterozygosity confirmed that the frequent loss of the wildtype allele in all tumours examined (Table 3). Analysis by PCR and southern blot does not detect point mutations which may account for the remaining 11% in the p53^{+/-}T380A mice and is currently being investigated. The detectable loss of both wild type alleles in most of the T380A mice was surprising. Most mammary tumors tested expressed the transgene (previous report, Table 3). Karyotypic analysis of all tumor cell lines established demonstrates considerable aneuploidy with

chromosome counts of 56-97 (normal count $2n=40$). Cells in culture also exhibit varying degrees of karyomegaly, are frequently multinucleate and are apparently genetically unstable in culture. Although chromosome painting has not directly contributed to LOH assignment, as planned it has enabled us to demonstrate instability in culture, which appears to be a phenotype of all the cell lines established. Despite this many of the established lines continue to express the transgene despite the absence of prolactin in the culture medium.

Summary

The data obtained in the present study support a role for cyclin E induced genomic instability in vivo. Furthermore, they suggest that maintaining the transgene may confer an growth advantage in vivo. The concept behind this study has been independently reported in a recent study, of Cyclin D, also implicated in mammary tumorigenesis. However, in $p53^{+/-}$ MMTV-cyclin D1 mice there was no enhancement of mammary tumorigenesis. Furthermore, ectopic expression of the cyclin D1 transgene was detected in the majority of non-mammary tumors which developed earlier in the $p53^{+/-}$ MMTV-cyclinD1 mice. In contrast, we observe increased tumorigenesis and no ectopic expression. Several lines of evidence indicate the cooperativity between deregulated cyclin E with p53 deficiency might be expected. Firstly, p53 null cells with deregulated cyclin E exhibit centrosome hyperamplification cyclin E which is a common observation in tumors and intimately linked to genomic instability. Secondly, it has been suggested that p53 forms a level of protection in cells with deregulated cyclin E and therefore loss of p53 would abrogate this mechanism of protection. This latter study also alludes to decreased cyclin E/Cdk2 kinase activity in cells overexpressing cyclin E, brought about by the induction of p21 by p53. In our study we have no evidence for p53 induction in the normal mammary epithelium of our mice. Furthermore, we see increased kinase activity in the midlactation mammary epithelia of T380A mice but not in those with the wildtype cyclin E allele. It is possible that this free kinase is the contributory factor in the induction of chromosomal instability in these mice, inducing lesions early many of which are subsequently lost with postlactation involution. Demonstration of these early genetic lesions, and a later loss of p53 heterozygosity, is important to test this hypothesis to explain the unaffected latency of tumorigenesis in these mice despite the dramatic increase in tumor penetrance.

Key Research Accomplishments

- Animal breeding complete
- All mice taken to the final time point
- All tissue collection and tumor analysis (as outlined in the original proposal) complete.
- Main manuscript in preparation pending final assay of interphase cytogenetics.

Reportable Outcomes

- Establishment of a new model of murine mammary tumorigenesis
- Demonstration of synergy between p53 heterozygosity and transgenic expression of the hyperstable form of cyclin E
- An incidental finding of hyperpigmentation in $Rb^{+/-}$ mice with pituitary pars intermedia lesions (Smith et al., Comparative Medicine 53:75-80).
- Application for joint position in as a murine oncopathologist and researcher in mammary tumor research (Medical Research Council, U.K.)

Conclusions

The initial objectives of this project have been met with few technical problems. Characterization of all tumors has been made with consultation of pathologists. More involved histopathological diagnoses have been presented at comparative pathology rounds held at TSRI in collaboration with Dr Kent Osborn (veterinary pathologist) and with consultation with Dr John Lee. The results obtained are largely as anticipated and in accordance with the hypothesis tested. The only obstacle encountered during these studies is accounting for the lack of decreased latency in mammary tumorigenesis. This is an obstacle that is inherent in targeting transgene expression to the mammary gland using promoters that are conditionally expressed during lactation. Indeed, discussions with researchers at the Era of Hope 2002 conference highlighted this issue as a number of researchers are using lactation specific promoters, such as beta lactoglobulin and whey acidic protein, as a means of conditionally expressing genes in vivo. Given that the mammary epithelia in the mouse almost fully involute post lactation this is an issue that should be considered in future studies and accounted for in study design. However, this should not preclude use of these models but should merely be anticipated with early detection of genetic lesions in midlactation mammary glands should be included in the original strategy adopted.

The results of this study demonstrate that deregulation of cyclin E (accompanied with cyclin E dependent kinase activity) is sufficient to cause genomic instability in vivo. The causes of deregulation of cyclin E are beginning to be elucidated. Indeed, findings by other workers in the mentor's laboratory (also funded by DOD breast cancer research fellowship, H. Strohmaier et al., *Nature* 2001, 413:316-22) have identified a novel tumor suppressor, the function of which is to degrade cyclin E, a loss of which directly leads to deregulation of cyclin E expression. Furthermore, it is likely that deregulation of expression, rather than simply gene amplification that results in genomic instability, in contrast to that of cyclin D where gene amplification appears to be a detrimental event.

During the execution of this project the PI has developed a keen interest in murine oncopathology, a field that is undermanned and essential for an understanding of the numerous models that are currently being developed. As a consequence, somewhat unanticipated at the initiation of the project, the PI is pursuing employment opportunities and formal qualification as a pathologist. During the course of the project (and not detracting from it) the PI has consulted in the phenotypic characterization of two further murine models developed within the mentor's laboratory which have resulted in an understanding of the roles of Cks1 and Cks2 in vivo (Spruck et al., 2001. *Mol. Cell*, 7:639-50; Spruck et al., *Science* 2003, 300:647-50).

References

- Bortner, D.M. and M.P. Rosenberg. 1997. Induction of mammary gland hyperplasia and carcinomas in transgenic mice expressing human cyclin E. *Mol Cell Biol* **17**: 453-9.
- Harrison, D.J., M.L. Hooper, J.F. Armstrong, and A.R. Clarke. 1995. Effects of heterozygosity for the Rb-1t19neo allele in the mouse. *Oncogene* **10**: 1615-20.
- Harvey, M., H. Vogel, E.Y. Lee, A. Bradley, and L.A. Donehower. 1995. Mice deficient in both p53 and Rb develop tumors primarily of endocrine origin. *Cancer Res* **55**: 1146-51.
- Jacks, T., A. Fazeli, E.M. Schmitt, R.T. Bronson, M.A. Goodell, and R.A. Weinberg. 1992. Effects of an Rb mutation in the mouse. *Nature* **359**: 295-300.
- Jacks, T., L. Remington, B.O. Williams, E.M. Schmitt, S. Halachmi, R.T. Bronson, and R.A. Weinberg. 1994. Tumor spectrum analysis in p53-mutant mice. *Curr Biol* **4**: 1-7.
- Kuperwasser, C., G.D. Hurlbut, F.S. Kittrell, E.S. Dickinson, R. Laucirica, D. Medina, S.P. Naber, and D.J. Jerry. 2000. Development of spontaneous mammary tumors in BALB/c p53 heterozygous mice. A model for Li-Fraumeni syndrome. *Am J Pathol* **157**: 2151-9.

- Lee, E.Y., C.Y. Chang, N. Hu, Y.C. Wang, C.C. Lai, K. Herrup, W.H. Lee, and A. Bradley. 1992. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature* **359**: 288-94.
- Park, M.S., J. Rosai, H.T. Nguyen, P. Capodieci, C. Cordon-Cardo, and A. Koff. 1999. p27 and Rb are on overlapping pathways suppressing tumorigenesis in mice. *Proc Natl Acad Sci USA* **96**: 6382-7.
- Smith, A. P. L.**, Henze, M., Osborn, K. G., Lee, J. A., Bortner, D. M., Rosenberg, M. P. and Reed, S. I. (2003) Switching of melanocyte pigmentation associated with pars intermedia tumors revealed in yellow pelage Rb^{+/-} and p27^{-/-} female mice. *Comparative Medicine*. 53(1):75-80.
- Spruck, C.H., K.A. Won, and S.I. Reed. 1999. Deregulated cyclin E induces chromosome instability. *Nature* **401**: 297-300.
- Spruck, C, Strohmaier, H., Watson, M., **Smith, A. P. L.**, Ryan, A., Krek, W. and Reed, S. I. (2001) A CDK-independent function of mammalian Cks1: targeting of SCF^{Skp2} to the CDK inhibitor p27^{Kip1}. *Molecular Cell*. 7(3):639-50.
- Spruck, C., de Miguel, M. P., **Smith, A. P. L.**, Ryan, A., Stein, P., Schultz, R. M., Lincoln, A. J., Donovan, P. J., and Reed, S. I. (2003) Cks2 is essential for metaphase/anaphase transition in mammalian meiosis. *Science*. 300(5619):647-50.
- Strohmaier H, Spruck CH, Kaiser P, Won KA, Sangfelt O, Reed SI. Human F-box protein hCdc4 targets cyclin E for proteolysis and is mutated in a breast cancer cell line. *Nature*. 2001 Sep 20;413(6853):316-22.

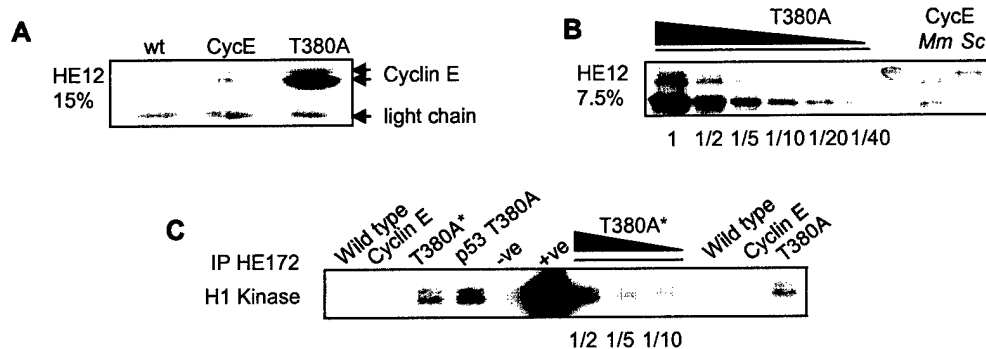


Figure 1. Transgene expressions and transgene dependent kinase activity in midlactation mammary glands. A) immunoblot analysis from wildtype, cyclin E and T380A 2 week post partum mammary glands with human cyclin E specific monoclonal antibody HE12. Note the transgene is expressed in two forms at 47 and 35kDa and that the faster migrating form is equally expressed in the cyclin E mice but is predominant in the T380A mice. B) titration of T380A transgene expression against that in the cyclin E mice (Mm) and a control protein in yeast (sc). Dependent upon comparison with the higher or lower migrating form, steady state expression is 5-20 fold higher in the T380A mice. C) Cyclin E dependent kinase activity is higher in T380A mice. Extracts from 2 week midlactation mammary glands were immunoprecipitated with HE172 human cyclin E-specific IgG. Histone 1 phosphorylation in the presence of radiolabeled ATP was greater in T380A mice than wild type mice (without the human cyclin E transgene) whereas in the cyclin E mice transgene dependent kinase activity was not elevated above wildtype. Kinase activity in p53^{+/+}-T380A mice was comparable to that in the T380A mice.

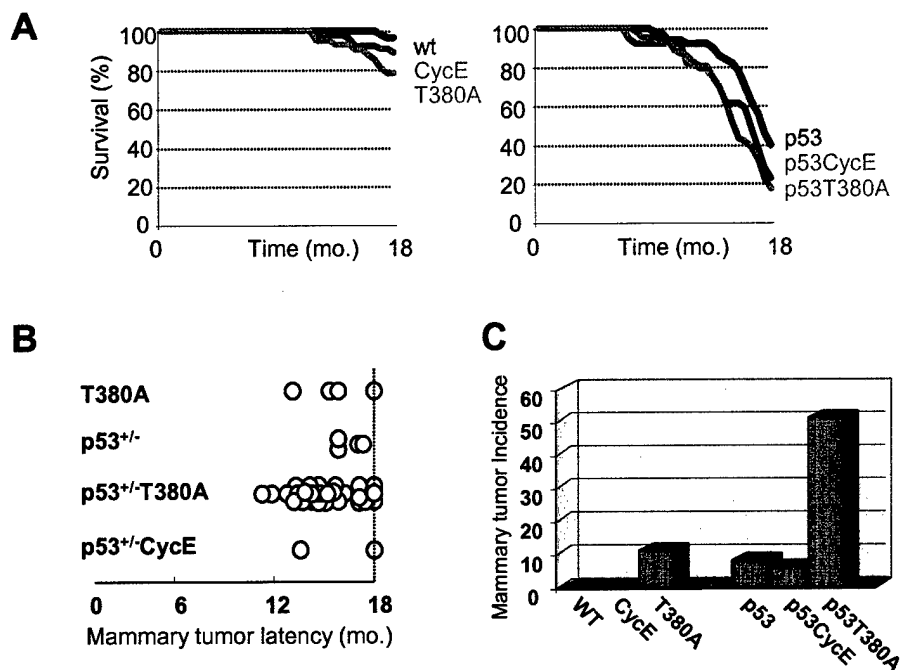


Figure 2. Survival curves and mammary tumor latency and penetrance. A) Mice expressing the transgene have moderately decreased life expectancy compared to the nontransgenic controls (left panel) and similarly modestly decrease survival in the p53^{+/+} lines. The difference is largely due to increased rates of mammary tumorigenesis. B) latency of mammary tumorigenesis was not significantly decreased in any particular genotype. C) synergy in mammary tumorigenesis in p53^{+/+}-T380A mice significantly above an additive effect from the levels observed in p53^{+/+} or T380A mice

Table 1. tumor incidence.

| Neoplasm | Genotype | wt | Rb ^{+/+} | Rb ^{+/-} CycE | Rb ^{+/-} T380A | p53 ^{+/-} | p53 ^{+/-} CycE | p53 ^{+/-} T380A | CycE | T380A |
|----------------------------------|----------|-----|-------------------|---------------------------|----------------------------|--------------------|----------------------------|-----------------------------|------|-------|
| Mammary carcinoma* | | - | - | - | - | 7% | 6% | 54% | - | 12% |
| Lymphoma ¹ | | 8% | - | - | - | 7% | 21% | 14% | 8% | 5% |
| Osteosarcoma | | - | - | - | - | 46% | 50% | 27% | - | - |
| Leiomyosarcoma | | 2% | - | - | - | 2% | - | 4% | 4% | - |
| Poorly differentiated sarcoma | | - | - | - | - | 11% | 3% | 10% | - | 3% |
| Pancreatic acinar cell | | - | - | - | - | 2% | - | - | - | - |
| Rhabdomyosarcoma | | - | - | - | - | 2% | - | 7% | - | - |
| Haemangiosarcoma | | 4% | - | - | 2% | 2% | - | 17% | 2% | 3% |
| Keratoacanthoma ² | | - | - | - | - | 2% | - | - | - | - |
| Pituitary ³ | | - | 100% | 92% | 76% | 7% | - | 2% | 2% | - |
| Medullary Thyroid carcinoma | | 2% | 92% | 89% | 90% | - | - | - | - | - |
| Harderian gland carcinoma | | - | - | - | - | - | 3% | - | - | - |
| Brochiolar/Alveolar ⁴ | | - | - | - | - | - | - | 4% | - | 6% |
| Glandular stomach carcinoma | | 2% | - | - | - | - | - | - | - | - |
| Tumor-free | | 88% | - | 5% | 4% | 24% | 16% | - | 84% | 75% |
| Number of mice | | 50 | 41 | 38 | 54 | 45 | 32 | 56 | 50 | 32 |

*Includes carcinoma in situ. Mice bearing more than 1 mammary lesion count as a single data point. ¹Includes thymic, splenic and generalized lymphomas. ²Keratoacanthoma of the pinna removed surgically, the mouse did not later develop further tumors. ³Neoplasia of the pars intermedia in Rb^{+/-} mice and pars distalis in the p53^{+/-} mice. ⁴Broncholar/alveolar carcinoma in 1 p53T380A mouse but adenoma in the remainder.

Table 2. Nuclei sizes in midlactation mammary glands

| | Genotype | | | | |
|-----------------------|-----------|-----------|-------------|--------------------------|-----------|
| | wildtype | p53 | T380A | p53 ^{+/-} T380A | CycE |
| Mean nuclei size (μm) | 6.74±1.01 | 6.55±0.74 | 5.05±0.98 * | 4.59±0.90 * | 6.29±0.84 |
| n | 288 | 233 | 230 | 371 | 204 |

*p<0.0001; ANOVA. Measurements were determined at 400x magnification from three separate tissue sections and pooled.

Table 3. Penetrance, p53 loss of heterozygosity and transgene immunoreactivity in mammary tumours.

| | Genotype | | | |
|------------------------------------|--------------------|------------|--------------------------|-------------------------|
| | p53 ^{+/-} | T380A | p53 ^{+/-} T380A | p53 ^{+/-} CycE |
| Incidence | 7% (3/45) | 12% (4/32) | 54% (30/56) | 6% (2/32) |
| p53 LOH ¹ | 100% (3/3) | 60% (3/5) | 89% (25/28) | 100% (2/2) |
| HE12 immunoreactivity ² | 0% (0/2) | 50% (2/4) | 84% (22/26) | 0% (0/2) |

¹Chromosomal region loss only, does not include point mutations. ²HE12 mouse monoclonal IgG recognizes human transgene only. Includes analysis of tumours that arose in mice later excluded from the study. Analysis for p53 point mutations in tumors not demonstrating clear LOH is ongoing.

Appendix

Exclusion/Inclusion For Statistics

40-70 mice were generated for each genotype and entered into program to obtain a minimum of 32 meeting inclusion criteria

Inclusion criteria

- 1) Homozygous at the transgene locus

Exclusion criteria

- 1) <2 pregnancies
- 2) Died/euthanised for non-neoplastic lesions
- 3) Died/euthanised prior to appearance of earliest mammary lesion

Number of mice meeting criteria

| <u>Transgene</u> | <u>wt</u> | <u>Tumour suppressor</u> | |
|------------------|-------------|--------------------------|--------------------------|
| | | <u>Rb^{+/-}</u> | <u>p53^{+/-}</u> |
| wt | 50/58 (86%) | 41/50 (82%) | 45/50 (90%) |
| CycE | 49/56 (87%) | 38/44 (86%) | 32/44 (73%) |
| T380A | 32/46 (70%) | 54/56 (96%) | 56/66 (84%) |

Exclusions

| <u>Genotype</u> | <u>Criterion1</u> | <u>Criterion2</u> | <u>Criterion3</u> |
|-----------------|-------------------|-------------------|-------------------|
| wt | 5 | 2 | 1 |
| CycE | 2 | 1 | 4 |
| T380A | 8 | 3 | 3 |
| Rb | 5 | 4 | - |
| RbCycE | 4 | 1 | 1 |
| RbT380A | - | - | 3 |
| p53 | 1 | 1 | 3 |
| p53CycE | 9 | 1 | 2 |
| p53T380A | - | 2 | 8 |

Where a mouse meets more than 1 exclusion criterion 1<2<3. Therefore a mouse listed as exclusion by criterion 3 is not excluded by 1 or 2

Mammary tumour stats

| <u>Transgene</u> | <u>wt</u> | <u>Tumour suppressor</u> | |
|------------------|------------|--------------------------|--------------------------|
| | | <u>Rb^{+/-}</u> | <u>p53^{+/-}</u> |
| wt | 0/50 (0%) | 0/41 (0%) | 3/45 (7%) |
| CycE | 0/49 (0%) | 0/38 (0%) | 2/32 (6%) |
| T380A | 4/32 (12%) | 0/54 (0%) | 30/56 (54%)** |

p>0.0008 by 2-tailed Fischer Exact Test